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Leucoagaricus ariminensis sp. nov., a lilac species from Italy

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Abstract—A new species, *Leucoagaricus ariminensis*, collected under *Cupressus sempervirens* in a park in Rimini (central Italy), is described and illustrated. The new species is compared with the other known lilac-coloured lepiotoid species, and its phylogenetic position, based on nrITS sequences, is determined. Colour photographs and illustrations of diagnostic anatomical characters are also provided.

Key words—*Agaricaceae*, *Agaricales*, lepiotaceous fungi, rDNA, taxonomy

Introduction

The genus *Leucoagaricus* Locq. ex Singer is considered to have a widespread geographical distribution even if it might appear more commonly linked to the tropical and sub-tropical zones of Africa and America (Singer 1986). Data in Vellinga (2004) indicate that the number of *Leucoagaricus* species in Europe increases as one moves southwards from the northern areas. Citations from the checklist of Italian fungi (Onofri et al. 2005) and other sources (Consiglio & Contu 2004, Zotti et al. 2008) establish occurrences of at least 52 *Leucoagaricus* species in Italy, a figure significantly higher than the numbers recorded in northern European areas (Vellinga 2004, Lange 2008). Of all species described in Italy, 58% occur in the north, 62% in the south (with islands included), and the majority, 73%, in the central regions. Considering only the non-

coastal northern regions, the number of species decreases to less than 40%, demonstrating the greater biodiversity of *Leucoagaricus* in milder climatic zones.

Numerous new species have been described in recent years (Ge 2010; Liang et al. 2010; Vellinga & Balsley 2010; Vellinga et al. 2010; Muñoz et al. 2012, 2014; Kumari & Atri 2013; Ye et al. 2014; Ge et al. 2015), a clear indication that the exact number of species in this genus is still incomplete. Some have been found in urban parks showing that these areas can be an interesting habitat for the study of this genus.

During a recent fungal biodiversity study conducted in Parco XXV Aprile, a public park in Rimini, a *Leucoagaricus* species was found, characterized by a dark violaceous pileus, violet/lilac tinged stipe and annulus rim, rather fleshy basidiomata, negative reaction to ammonia vapours, and white flesh that yellowed when handled. Comparison with the known *Leucoagaricus* species and ITS sequence analyses indicated that this species represents a novel taxon, which we describe below.

Materials & methods

Morphology

The macroscopic descriptions are based on observations of fresh material, which was photographed with a Canon EOS 60D camera fitted with a Canon EF-S 60 mm macro lens. The specimens were dried with an electric dryer. Voucher specimens were deposited in the Herbarium, Museo di Storia Naturale di Venezia, Venice, Italy (MCVE). The terminology used in the descriptions follows Vellinga (2001). Basidioma colours were coded according to Munsell (1994). The micro-morphological characters are based on the study of dried material rehydrated in KOH or NH_4OH; other reagents used were Congo Red for staining cell-walls, anionic phloxine for colouring the cytoplasm, Cresyl Blue for testing the metachromatic reaction of the spores, Melzer's reagent to detect spore dextrinoid reactions, glycerine tampon L4 for the observation of pigments, Chlorazol Black (L4C) for contrasting the septa. Spore nuclei were stained with DAPI (1 mg/mL) and observed with a fluorescence microscope. All observations and measurements were carried out with an optical trinocular Optrech B5 microscope, with ×10, ×25, ×40, ×60 lenses and a ×100 immersion lens. Microphotos were taken with a Canon EOS 60D camera connected to the microscope with an extension tube. At least 20 basidia, basidiospores, cystidia and terminal elements of the pileipellis were measured. Basidiospores were measured both from fresh spore deposit and from dried specimens. Basidiospore dimensions are expressed as (a) b–c (d), where (a) = minimum value, b = (average – standard deviation), c = (average + standard deviation), and (d) = maximum value. The following abbreviations are used: L = number of lamellae, and l = number of lamellulae; [x/y/z] indicates that altogether x spores, from y samples, from z collections were measured.

DNA extraction, PCR amplification, DNA sequencing

The DNA was extracted with NaOH (Osmundson et al. 2013); 2 mg of dry sample were homogenized in 250 µl of 0.5M NaOH with a pestle. After 10 minutes to allow for sedimentation, 5 µl of the extract was removed and diluted in 195 µl of 100mM Tris-HCl at pH 8.0, and 1 µl of the dilution was used as template DNA. The ITS region was amplified with primers ITS1F (Gardes & Bruns 1993) and ITS4 (White et al. 1990). PCR was performed in a 25 µl reaction volume following Gardes & Bruns (1993).

PCR products were purified and sequenced by IGA Technology Services (Udine, Italy).

Sequence alignment & phylogenetic analysis

Sequences were assembled and edited in Geneious v. 8.1.2 (Kearse et al. 2012) and then submitted to GenBank (see Fig. 1 for accession numbers).

The initial BLASTn results revealed that all reference sequences with identities >85% (and E = 0) to *L. ariminensis* belong to the Piloselli clade, following criteria reported in Vellinga (2010), Vellinga et al. (2010, 2011), and Muñoz et al. (2012, 2014).

Our dataset includes sequences in /Piloselli from previous phylogenetic studies (Vellinga 2010; Vellinga et al. 2010, 2011; Munoz et al. 2012, 2014) and other sequences with greatest similarity available in GenBank. We also included a sequence of *L. ionidicolor* (sequenced in this work, KU953373) collected in the Czech Republic and described in Holec (2009) and added for comparison six sequences representing *Leucoagaricus* sect. *Rubrotincti. Cystolepiota seminuda* was used as outgroup.

The sequences were aligned using MAFFT v 7.017 (Katoh et al. 2002) with default conditions for gap openings and gap extension penalties.

Phylogenies were inferred with RAxML v.7.2.8. (Stamatakis 2006) and MrBayes v. 3.2.4 (Huelsenbeck & Ronquist 2001) using jModelTest v. 2.1.7 (Darriba et al. 2012) to choose best-fit models of nucleotide substitution. The GTR+G model was chosen for both analyses.

A total of 1000 bootstrap replicates were performed to assess the relative robustness of the branches of maximum likelihood in RAxML. In Bayesian analysis, MCMC was performed for four chains and run for 1,0000,000 generations (sampling trees every 1000 generations) with a 25% burn-in.

Taxonomy

The alignment includes 96 ITS sequences representing 28 species of white-spored *Agaricaceae* and comprises 805 characters.

Both Maximum Likelihood and Bayesian analyses produced the same topology. Only the Bayesian tree with posterior probability and bootstrap values is shown (Fig, 1). *Leucoagaricus ariminensis* is a sister species of *Leucoagaricus ionidicolor* (MLB = 100; BPP = 1), with which it shares 91.7% of bases/residues. They form the sister clade of *Lepiota decorata* and are placed within /Piloselli. The sequence of *L. ionidicolor* from the Czech Republic specimen shares 658/663 (99%) nucleotides compared with the Dutch sequence (AY 176415).

Fig. 1. Bayesian phylogram obtained from the general ITS sequence alignment of *Leucoagaricus*/*Lepiota* spp.; *Cystolepiota seminuda* was used as outgroup taxon. Only BPP values >0.95 (in bold) and MLB values >50% are shown above clade branches. Sequences of *Leucoagaricus ariminensis* are in bold.

Fig. 2. *Leucoagaricus ariminensis* (holotype, MCVE 28443). Basidiomata. Scale bar = 20 mm.

Leucoagaricus ariminensis Dovana, Angeli, Contu & Brandi, **sp. nov.** Figs 2–4

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Differs from *Leucoagaricus ionidicolor* by its more robust basidiomata, stipe that is usually shorter than the pileus diameter, more variably shaped and larger cheilocystidia, larger spores, and longer basidia.

Type: Italy, Emilia-Romagna, Rimini (RN), Parco XXV Aprile, along an avenue of cypress trees, 27.12.2014, legit L. Brandi (**Holotype**, MCVE 28443; GenBank KU953370).

Etymology: the specific epithet "*ariminensis*" derives from Ărīmĭnum, Latin name for Rimini, a city in Emilia Romagna Region of the Italian Republic.

Pileus 3.5–8 cm, initially convex, becoming plano-convex, then depressed with age and plane in the centre; margin regularly involute at first, becoming incurved for a long time, then finally extended and somewhat irregularly sinuate; violet grey, violet brown-grey, violet (5P 3/4–8; 5P 4/4–8), tomentose, with tufts of thick hairs forming fine, densely packed squamules (lens), sparser at the margin and there the white flesh showing through. Negative reaction to aqueous ammonia.

Stipe 2–4(–4.5) × 1–2 cm, cylindrical, shorter than the pileus diameter, tapering upwards from an enlarged but not bulbous base, tapering downwards in some specimens, base well-covered with leaf-mould or debris, fibrous, fistulous; surface covered with white hairs faintly tinged with violet (5P 7/2-4)

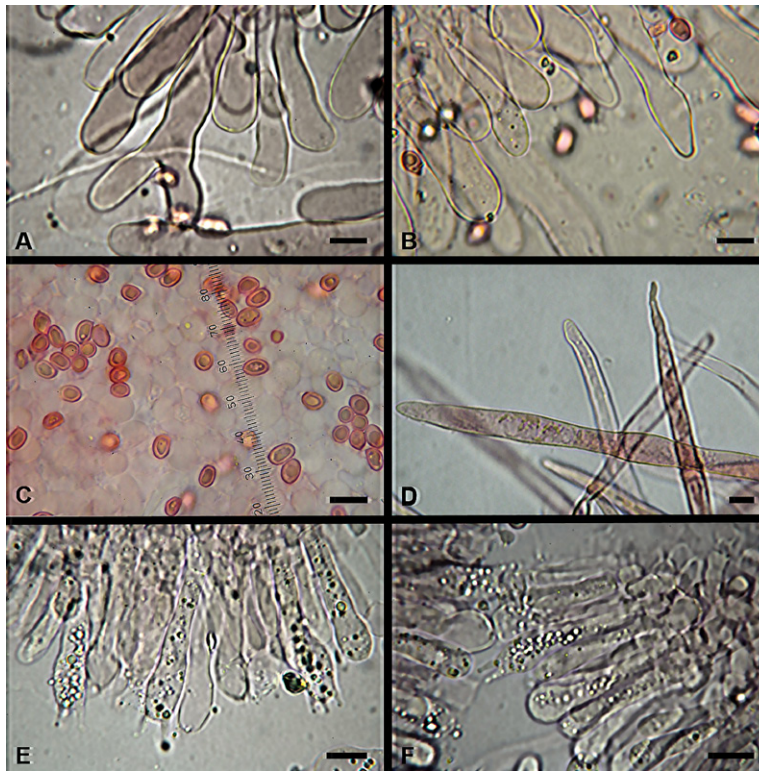

Fig. 3. *Leucoagaricus ariminensis* (holotype, MCVE 28443). A, B. Cheilocystidia. C. Spores. D. Terminal elements of the pileipellis. E. F. Basidia. Scale bars = 10 µm.

above the annulus, whitish and stained purple below (5RP 5/2–4)) where brown stains appear with age or handling. Annulus pendant, membranous, tomentose on the underside like the stipe surface, rim concolorous with the pileus.

Lamellae, L = 70–90, l = 2–3, moderately crowded, rounded and free at the stipe with a collarium, interspersed with numerous truncate lamellulae of various lengths; white to pale cream; edge sterile.

Flesh thick, tender, fragile, fibrous in the stipe, yellowing when handled or with age; odour faint, acidulous, only perceptible in young specimens, becoming unpleasant and smelling of fish with age; taste mild. Non-staining in contact with ammonia. Spore deposit light cream (2.5YR 8/1).

Basidiospores [270/3/4] (5.5–)6.5–8.1(–10.5) × (3.3–)4.1–4.9(–6.1) µm, on average 6.9–7.8 × 4.3–4.6 µm, Q = (1.18–)1.45–1.84(–2.31) Q_{av} = 1.5–1.8;

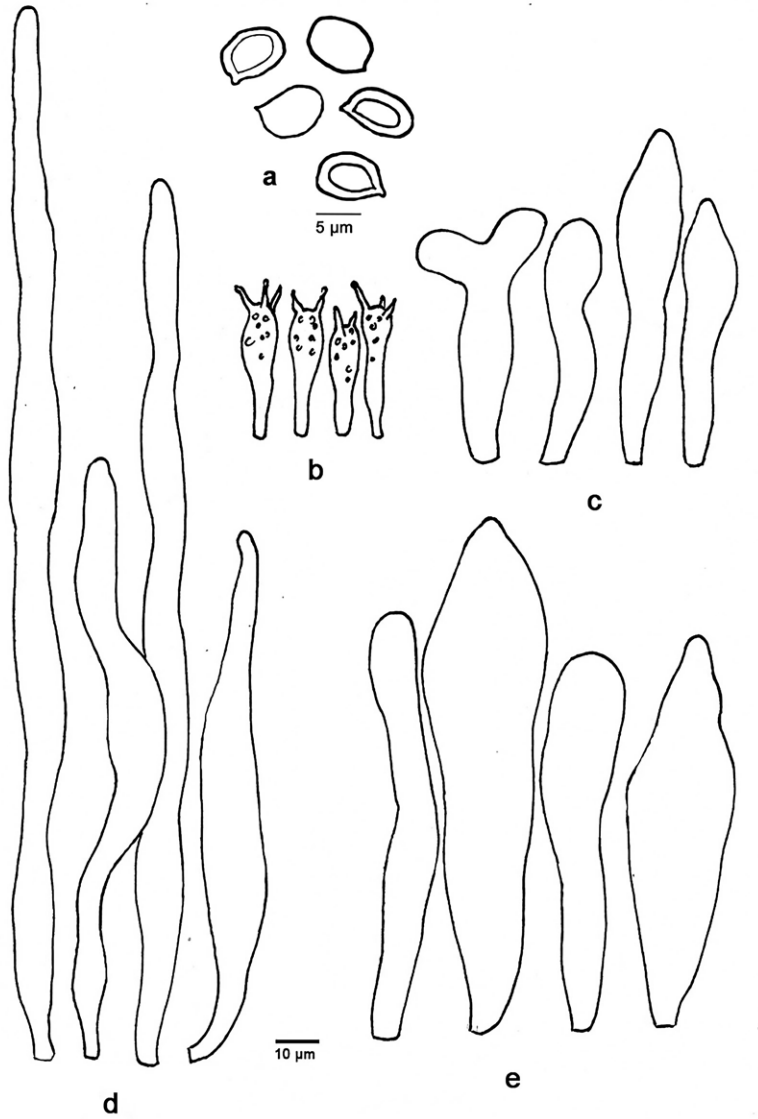

Fig. 4. *Leucoagaricus ariminensis.*
a Spores. b Basidia. c Cheilocystidia. d. Terminal elements of the pileipellis. e. Caulocystidia.
Scale bars: a = 5 µm; b–e = 10 µm. (Drawings by Maria Tullii).

mainly ellipsoid, with a rounded or sometimes subacute apex, some amygdaliform or subcylindrical, smooth, guttulate, thick-walled, without a germ pore or suprahilar depression, dextrinoid, metachromatic in Cresyl Blue, binucleate (rarely tetranucleate). Basidia 33.9–38.6 × 7.0–8.8 µm, 4-spored, some 2-spored, seldom 1-spored, at times somewhat swollen in the upper part. Lamellar trama ± regular, hyphae 5–10 µm wide, without clamps. Pleurocystidia not observed. Cheilocystidia 48–73 × 12–15 µm, abundant, variable in shape, from cylindrical, clavate with an obtuse apex to lageniform, rarely bifurcate. Pileipellis formed by repent interwoven hyphae, becoming a trichoderm near the squamules, with long, apically rounded terminal elements intermingled with elements that are tapered towards the apex, 150–330 × 7–12 µm; vacuolar pigment brownish-green-grey, at the centre some hyphae with granular vacuolar pigment; greenish-yellow excretory hyphae present. Non-gelatinised hyphae at disc. Subpellis formed by ± parallel hyphae, 9–16 (20) µm wide, slightly interwoven, with apically rounded, clavate and cylindrical terminal elements. Caulipellis formed by hyphae 5.9–13.8 µm wide, ± parallel, the outer ones interwoven with apically rounded, clavate and cylindrical terminal elements, some hyphae ending with long hairs; branched hyphae and some with excrescences also observed; caulocystidia lageniform, cylindrical, clavate, trunco-conical, 74–130 × 11–40 µm. Clamps absent.

Ecology: gregarious on bare sandy soil rich in vegetable litter (and animal excrements) under old cypress trees, *Cupressus sempervirens* L. In winter, from a single locality in Emilia Romagna.

Additional material studied: **ITALY**, **Emilia-Romagna**, **Rimini** (RN), Parco XXV Aprile: along an avenue of cypress trees, 12.12.2014, legit L. Brandi (MCVE29052; GenBank KU953372); 15.01.2015 legit A. Brandi (MCVE29053; GenBank KU953371).

Discussion

Leucoagaricus ariminensis has, as major morphological features, a rather fleshy predominately violaceous basidioma, usually with a stipe shorter than the pileus diameter and with a membranous purple-rimmed white annulus. It is distinguished by spores without a germ pore and a non-gelatinous pileus covering with somewhat elongated terminal elements that at times taper towards the apex. Since the basidioma surfaces in *L. ariminensis* react negatively to ammonia, this species would be placed in *Leucoagaricus* subsect. *Trichodermi* Bon & Migl. according to Bon's (1993) classification. However, our molecular results do not support this attribution.

ITS sequence analyses support *L. ariminensis* in *Leucoagaricus* sect. *Piloselli* (Kühner) Singer, indicating that a negative reaction to ammonia is not a constant within this section.

In both Bayesian (Fig. 1) and ML trees, *L. ariminensis* is distinct from *L. ionidicolor* Bellù & Lanzoni from the Netherlands (AY176415) and the Czech Republic (KU953373). Statistics support this separation (BPP = 1, MLB = 100%), and sequences from both *L. ionidicolor* collections differ from *L. ariminensis* by at least 42 nucleotides.

The ITS sequence from the Czech *L. ionidicolor* basidioma differs from that of the Dutch basidioma by only 5 nucleotides. The Czech specimen has "terminal cells which are narrowly clavate when young, then very long, 80–200 × 15–20 µm narrowly fusiform with rostrate apex" (Holec 2009), unlike the pileipellis of *L. ariminensis* where no rostrate elements are present.

Compared to *L. ariminensis, L. ionidicolor* has a more slender stem, a minutely squamulose pileus surface, and shorter basidia, (14–21 × 7–8 µm, Bellù & Lanzoni 1988; 14–21 × 5.5–6.5 µm, Vellinga 2001), smaller, ellipsoid to oblong basidiospores ((5–)5.5–6.5 × (3–)3.3–3.7(–4) µm, Bellù & Lanzoni 1988; 6.0–7.0 × (3.5–)4.0–4.5 µm, Vellinga 2001), and smaller clavate cheilocystidia (23–45 × 8–11(–12) µm, Bellù & Lanzoni 1988; 23–40 × 8.0–10 µm often with curved pedicel, Vellinga 2001). By contrast, the *L. ariminensis* spores range from ellipsoid to oblong with a rounded apex, are rarely amygdaliform, and show a great variability with regard to their dimension and Q within different basidiomata.

Leucoagaricus ionidicolor var. *major* J. Charb. et al., which differs mainly from the type species by its larger pileus and deeper shade of violet / purple, is very similar to *L. ariminensis* from which it can be distinguished by its slightly smaller spores (6–7 × 4–4.5(5) µm), shorter (100(–150) µm) fusiform terminal pileipellis elements, and smaller and more uniformly shaped cheilocystidia (Bon & Charbonnel 2000; no basidium dimensions provided). Further molecular studies are needed to clarify the position of this variety within the *L. ionidicolor* group.

Bon (1993) placed two more violaceous-coloured species in *Leucoagaricus* subsect. *Trichodermi: L. ianthinophaeus* Locq. and *L. ianthinosquamulosus* Guinb., both described from France.

Leucoagaricus ianthinophaeus, still not well-known possibly because of its rarity, can be distinguished from *L. ariminensis* by its slender white glabrous stipe with no trace of an annulus, smaller spores, inconspicuous cheilocystidia, and its pileus covering composed of much shorter hyphae with terminal

elements 30–50 × 5–10 µm according to Bon (1993), who re-examined Locquin's original material. *Leucoagaricus ianthinosquamulosus* can easily be distinguished by its smaller basidiomata, definitely larger subfusiform spores (6.5–10(–12.5) × 4–5(–6) µm), shorter (16–50 µm) and more variably shaped cheilocystidia, and encrusting pigments in the pileus covering (Bon 1993, Guinberteau 1993).

Leucoagaricus idae-fragum Guinb. et al., a pink European species synonymized with *Lepiota decorata* based solely on morphology by Vellinga (2006), differs mainly in its remarkable raspberry red cap colours, the presence of a persistent general veil, a green ammonia reaction on gills and cap, and the differently shaped cheilocystidia (Guinberteau et al. 1998).

Lepiota roseilivida Murrill from western North America (= *Leucoagaricus marriageae* (D.A. Reid) Bon from Europe) can be diagnosed immediately by its slender appearance, spores 6.7–9.8 × 3.8–5.7 µm that are oblong or amygdaliform in profile, and green-staining reaction of the external basidioma surfaces to ammonia (Vellinga 2006).

Leucoagaricus brunneolilacinus Babos is a brownish lilac species with a negative ammonium reaction. It becomes distinctly orange when bruised and differs mainly from *L. ariminensis* by smaller spores (4–)4.2–5.1(–5.4) × 2.3–3.2(–3.5) µm, shorter (13–26 µm) cylindrical to clavate cheilocystidia, and a pileipellis with shorter (6–60 × 5–12(–16) µm) clavate terminal elements that in young basidiocarps are intermixed with scattered globose cells (Candusso & Lanzoni 1990).

Two other *Leucoagaricus* species with lilac basidiomes, *L. purpureolilacinus* Huijsman from Europe and *L. subpurpureolilacinus* Z.W. Ge & Zhu L. Yang from Asia, are phylogenetically well separated from /Piloselli and differ from *L. ariminensis* mainly by their clavate cheilocystidia crowned with crystals and different pileipellis (Vellinga 2001, Ge et al. 2015).

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